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Novel, High Throughput Method to Study in Vitro Protein Release from Polymer Nanospheres

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Controlled delivery of therapeutic protein drugs using biodegradable polymer carriers is a desired characteristic that enables effective, application-specific therapy and treatment. Previous studies have focused on protein delivery from polymers using conventional “one-sample-at-a-time” techniques, which are time-consuming and costly. In addition, many therapeutic proteins are in limited supply and are expensive, so it is desirable to reduce sample size for design and development of delivery devices. We have developed a rapid, high throughput technique based on a highly sensitive fluorescence-based assay to detect and quantify protein released from polyanhydrides while utilizing relatively small amounts of protein ($\sim 40 \mu\text{g}$). These studies focused on the release of a model protein, Texas Red conjugated bovine serum albumin, from polyanhydride copolymers based on sebacic acid (SA) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH). The protein release profiles were assessed simultaneously to investigate the effect of polymer device geometry (nanospheres vs films), polymer chemistry, and pH of the release medium. The results indicated that the nanosphere geometry, SA-rich chemistries, and neutral pH release medium led to a more rapid release of the protein compared to the film geometry, CPH-rich chemistries, and acidic pH release medium, respectively. This high throughput fluorescence-based method can be readily extended to study release kinetics for other proteins and polymer systems.

Introduction

The delivery of protein-based drugs (such as vaccine antigens, therapeutic proteins, and growth factors) using biodegradable polymeric devices has become an extensively explored area of innovation and research over the past decade.^{1–5} Often, the dual tasks of providing an environment capable of maintaining the functionality of the protein drug and releasing it in a sustained manner are challenging. In addition, the assays available for protein release kinetics are limited in that they lack sensitivity and are resource consuming. Many current protein release systems are carried out in a conventional “one-sample-at-a-time” format and require several milligrams of protein for adequate detection and quantification.

Several degradable polymeric biomaterials have shown much potential as protein carriers including polyesters, polyorthoesters, and polyanhydrides.^{3,6–13} Polyester-based systems undergo bulk erosion allowing significant water ingress and increased microenvironment acidity, which may affect both the stability of the protein and its release kinetics. Polyanhydrides, which are generally more hydrophobic, undergo surface erosion through hydrolytic degradation, which makes the erosion more controllable, allowing for tailored degradation ranging from days to months.^{14–16} In all these systems, it is evident that the chemistry of the polymeric carrier plays a significant role in maintaining the function of the protein and in governing its release kinetics.

This work is focused on polyanhydrides, which are biocompatible materials that have been studied extensively for use in drug and vaccine delivery.^{1,3,14,17–28} The polymers of interest are based on 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and sebacic acid (SA). In addition to their biocompatibility, the degradation of these materials is base-catalyzed^{6,29} and can be tuned allowing for drug delivery ranging from weeks (SA-rich chemistries) to months (CPH-rich chemistries).^{2,14,15}

In this design of delivery vehicles, one can envision a large parameter space to investigate, based on the chemistry of the polymer, the type of protein drug to be encapsulated, the desired release rate of the protein, the in vivo release environment of the protein, the required geometry of the protein encapsulation device (nanospheres for drug/vaccine delivery or films for drug-eluting implants), and the end-use application. High throughput approaches, employing combinatorial library synthesis, can be valuable to more rapidly develop and/or screen new biomaterials for controlled delivery of proteins. There are several examples in the literature of high throughput screening of biomaterials to study their interactions with proteins and cells.^{4,24,26,30–40} Vogel et al. developed the first high throughput approach to study combinatorial drug release kinetics from CPH:SA polyanhydride films.²⁷ In this study, the release profile of ethidium bromide bisacrylamide from polyanhydride films was investigated, and it was shown that the more hydrophobic (CPH-rich) chemistries released the dye the slowest. The conditions used to study the release of dyes, which can withstand harsh processing (e.g., high temperature, solvent exposure, low vacuum, etc.), are not ideal for protein-based

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drugs. In addition, some of the colorimetric methods available to quantify dyes are not readily applicable to protein analysis.

In this work, the development of a novel and multiplexed technique to concurrently study the release kinetics of proteins from polyanhydrides with multiple varying parameters (device geometry, polymer chemistry, and pH of release medium) by employing a highly sensitive fluorescence-based assay is described. To the best of the authors' knowledge, no combinatorial methods are currently available to evaluate the delivery of protein-based drugs from polymers. The high throughput technique described herein allowed for the rapid detection and quantification of a model fluorescent protein (Texas Red bovine serum albumin (TRBSA)) released from five different chemistries of two different CPH:SA polymer device geometries (nanospheres and films) in media of three different pHs. This highly sensitive, fluorescence-based approach reduced the amount of protein needed (total of $\sim 40 \mu\text{g}$) because very small amounts of the fluorochrome-conjugated protein are necessary to get sufficient excitation, emission, and quantification as compared to alternate assays, which require significantly larger amounts of protein to obtain similar data. In addition, this novel method eliminated the need for repeated sampling, which can often introduce experimental error, and allowed for combinatorial protein release and simultaneous, rapid protein detection and quantification. High throughput methods are an invaluable resource for studying release of protein-based drugs, which are often made recombinantly and are therefore expensive and available in limited supplies. The use of multiplexed methods to study protein release kinetics will help to more readily advance the rational design and optimization of protein-based drug and vaccine delivery systems.

Materials and Methods

Materials. The chemicals utilized in monomer synthesis, 1-methyl-2-pyrrolidinone, 4-*p*-hydroxybenzoic acid, and 1,6-dibromohexane were purchased from Sigma Aldrich (St. Louis, MO); and sulfuric acid, was obtained from Fisher Scientific (Fairlawn, NJ). The chemicals needed for the polymerization, nanosphere fabrication, and buffer preparation include acetic anhydride, chloroform, methylene chloride, petroleum ether, pentane, monobasic potassium phosphate, dibasic potassium phosphate, sodium acetate trihydrate, and glacial acetic acid, all of which were purchased from Fisher Scientific. BSA was purchased from Sigma Aldrich. The microbicinoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, IL). Texas Red conjugated BSA was obtained from Invitrogen (Carlsbad, CA). Deep-welled 96-well polypropylene (0.5–2.0 mL) plates and sealing mats were purchased from Corning (Corning, NY).

Polymer Film Library Synthesis and Characterization. The CPH monomer was synthesized as described previously.²⁸ The SA monomer was purchased from Sigma Aldrich. CPH:SA copolymer libraries were synthesized from the corresponding monomers via a melt polycondensation reaction in multiwell substrates utilizing a robotic deposition apparatus, as reported previously.^{4,25} Copolymer chemistry and molecular weight were determined for the polymer film

libraries by proton nuclear magnetic resonance (^1H NMR) spectroscopy using a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Each sample was dissolved in deuterated chloroform, and the chemical shifts were calibrated with respect to the chloroform peak ($\delta = 7.26$ ppm). Gel Permeation Chromatography (GPC) was also used to measure the molecular weight on the polymer film libraries. Samples were dissolved in HPLC-grade chloroform and separated on a Waters GPC chromatograph (Milford, MA) containing PL Gel columns (Polymer Laboratories, Amherst, MA). Elution times were compared to monodisperse polystyrene standards (Fluka, Milwaukee, WI). The surface chemistry of the combinatorially synthesized CPH:SA polymer film libraries (no protein) was evaluated at high throughput using Fourier transform infrared spectroscopy with a Nicolet 6700 FTIR spectrometer (Thermo Scientific) as described previously.^{4,26}

Fabrication of Protein Encapsulated Film and Nanosphere Libraries and Nanosphere Library Characterization. An automated polymer synthesis/nanosphere fabrication device was designed for the initial deposition of the monomer library into a multivial substrate. Following synthesis, the combinatorial nanosphere library was fabricated from the polymer film library as described before.^{4,26} Additional steps were incorporated into the process for protein encapsulation. The protein (TRBSA) was initially homogenized (Tissue-Tearor, Biospec Products, Bartlesville, OK) in chloroform for 60 s at 10,000 rpm resulting in a final concentration of 1 mg/mL. The protein/chloroform solution was robotically deposited via syringe pumps into each vial of the multivial film library, thus dissolving the polymer films with a resulting polymer concentration of approximately 25 mg/mL. Each solution was homogenized for 60 s at 10,000 rpm to uniformly disperse the polymer and the protein in the chloroform. They were either dried to create the protein encapsulated film library (in a deep well, clear, polypropylene 96-well plate) or precipitated into petroleum ether and dried to create a protein-encapsulated nanosphere library. Smaller quantities of the protein-encapsulated nanospheres were weighed out and transferred to a deep-welled, clear, polypropylene 96-well plate for evaluating the release kinetics. This nanoprecipitation process is a modification of a previously described method.^{4,41} Scanning electron microscopy (SEM) was performed using a JEOL JSM-840A SEM (JEOL USA Inc., Peabody, MA) to study the external morphology of the protein-loaded nanospheres by coating their surface with 200 Å of gold.

TRBSA Release Kinetics and Protein Quantification. Following fabrication of the protein-loaded film and nanosphere libraries, 1 mL of the appropriate buffer (phosphate buffer pH 7.3, phosphate buffer pH 6.0, or acetate buffer pH 4.3) was added to each well. The well plates were sealed to prevent evaporation and incubated in a horizontal shaker at 37 °C and 100 rpm for the duration of the experiment. TRBSA detection and quantification was performed incrementally throughout the study, which was terminated after 1 month. The protein release data is presented as a cumulative fraction of protein released, in which the amount of protein released is normalized by the total amount of protein

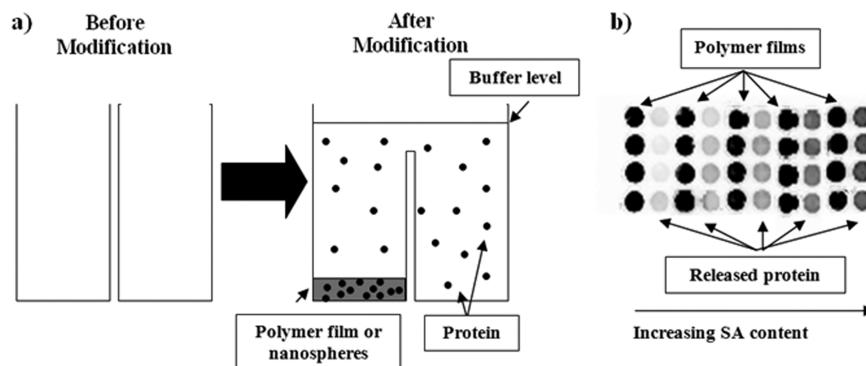


Figure 1. (a) Schematic of modifications made to horizontal pair of wells in the 96-well polypropylene release plate, and (b) actual fluorescence images of the 96-well plate while performing the TRBSA release studies from CPH:SA films (only wells with TRBSA in them are visible in the image).

encapsulated into the nanospheres or films. The TRBSA release was quantified using two methods: a micro-BCA assay and a high throughput fluorescence-based assay.

Microbicinchnonic Acid (BCA) Assay. Three hundred microliter samples were withdrawn at several time points from each release well and quantified with the micro-BCA assay. Samples were run in triplicate, as described by the manufacturer (Pierce). Fresh buffer was added to the sample well to maintain constant sink conditions.

High Throughput Fluorescence-Based Assay. In this automated technique (i.e., no sampling), each clear, deep-welled, polypropylene 96-well plate was modified by joining each neighboring pair of wells between neighboring columns: 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12 (i.e., wells A1 and A2, B1 and B2, A3 and A4, etc. were joined together) (Figure 1). The first wells were home to the fluorescent protein-loaded film or nanospheres, and the second, adjoining wells were empty. A 500 μ L portion of buffer was added to all the wells, and the plate was centrifuged (100 rcf for 10 min) to localize the nanospheres to the bottom of the wells. The wells were slowly filled to the top with buffer, and because of the modified well geometry, any released protein was uniformly dispersed between the two wells while keeping the films and nanospheres isolated to the first well. Each subsequent time prior to protein quantification, the polymer libraries were centrifuged (100 rcf for 10 min) to localize the nanospheres and film particulates to the bottom of the well. High throughput protein detection was performed with a Typhoon 9410 imaging system (GE Healthcare, Piscataway, NJ). The 96-well plate was placed on a flatbed scanner, and a laser was directed at the bottom of the plate to excite the fluorochrome-conjugated protein. The light emission was collected through a series of filters, which was immediately output to a computer quantification program (Image Quant TL, GE Healthcare, Piscataway, NJ). A row of protein standard concentrations was included in the 96-well plate to account for the effect of subsequent scanning or light bleaching on the fluorescent protein.

Results/Discussion

In this work a novel, fluorescent, high throughput technique for studying protein release has been designed, enabling rapid data quantification in a multiplexed format,

Table 1. Molecular Weight Analysis of CPH:SA Copolymer Film Libraries Using GPC and ^1H NMR

CPH:SA polymer library	M_n (Da) from GPC	M_n (Da) from ^1H NMR
Poly(SA)	11154	12555
25:75 CPH:SA	9692	10854
50:50 CPH:SA	13264	12872
75:25 CPH:SA	12674	13442
Poly(CPH)	16477	15247

eliminating error associated with repeated sampling, and minimizing sample size requirements. This approach allowed for the simultaneous evaluation of several key parameters involved in protein release including polymer chemistry, device geometry, and pH of release medium, which were carried out using two modified 96-well release plates.

Characterization. The CPH:SA film libraries were characterized with high throughput FTIR to determine surface chemistry and the overall accuracy of the deposition process. The results demonstrated excellent agreement between the intended molar compositions deposited and the actual molar compositions deposited into the multiwell substrate using the automated deposition apparatus (data not shown). These findings are consistent with previous work^{4,26} and confirm the accuracy of the depositions apparatus. In addition, molecular weight and copolymer composition were determined with ^1H NMR and GPC, Table 1, and the results were in agreement with conventionally synthesized CPH:SA copolymers.^{15,42} Following characterization of the polymer film libraries, the TRBSA loaded nanosphere libraries were characterized with SEM to determine shape and size. They were found to be very similar to previously published results with polyanhydride nanospheres^{4,41} with an average size of ~ 300 nm. Representative images from two selected chemistries are shown in Figure 2.

Combinatorial Protein Release from Polyanhydrides Nanospheres. Knowledge of the protein release kinetics from these carrier systems is very important for the rational design and optimization of devices for in vivo applications. To investigate this, the effect of polyanhydride nanosphere chemistry on TRBSA release was evaluated at high throughput using the highly sensitive fluorescence-based assay. The combinatorially measured protein release kinetics was validated with a commonly used micro-BCA assay. The multiplexed method enabled five different nanosphere chemistries

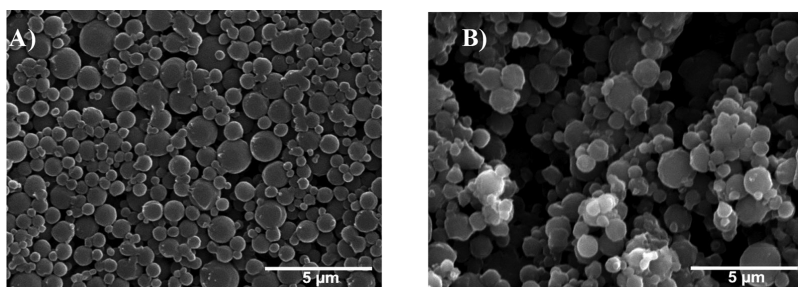


Figure 2. SEM images of TRBSA-loaded polyanhydride nanospheres: (A) poly(SA) and (B) 50:50 CPH:SA.

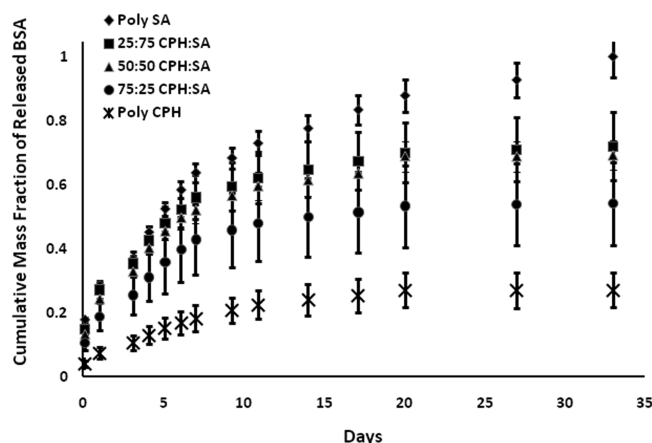


Figure 3. Cumulative mass fraction of BSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 7.3 as detected by the micro-BCA assay. Error bars represent standard deviation and $n = 4$.

ranging from 100 mol % SA to 100 mol % CPH to be simultaneously evaluated in replicates of four. The polymer chemistry played an integral role in controlling the release of TRBSA from the CPH:SA nanosphere library with the most hydrophobic chemistry (i.e., poly(CPH)) releasing the protein the slowest and the least hydrophobic chemistry (i.e., poly(SA)) releasing the protein the most rapidly (Figure 3). It is well-known that polymer hydrophobicity directly influences polymer erosion and protein release kinetics.^{14,18,42} This high throughput method allowed for other observations to be made simultaneously, which were consistent with previous work, including complete release of TRBSA from poly(SA) nanospheres¹⁸ and initial protein bursts of 5–20% (nanosphere chemistry dependent).¹⁵ As discussed previously, the TRBSA release profile obtained with the fluorescence-based assay was validated with the micro-BCA assay, which yielded consistent protein release profiles for all the CPH:SA nanosphere chemistries tested (Figure 4). This provides evidence supporting the accuracy and reliability of the high throughput fluorescence-based assay. In fact, in most cases the micro-BCA assay demonstrated more variability in the release curve data than the fluorescence-based assay, which is likely a result of error introduced with repeated sampling and the use of a less sensitive protein detection assay. The results indicate that this method would be amenable to study protein release kinetics in other biodegradable polymer systems intended for drug or vaccine delivery. This technique can also be used to study protein release under other simulated *in vitro* conditions that better mimic *in vivo* applications (e.g., in the presence of serum proteins).

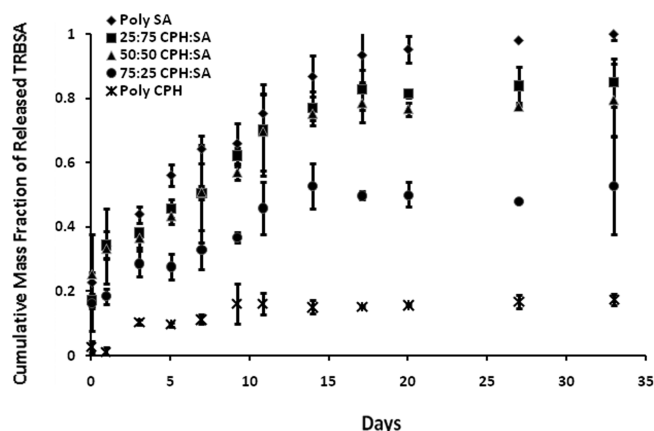


Figure 4. Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 7.3 as detected by the high throughput fluorescence assay. Error bars represent standard deviation and $n = 4$.

It is known that protein release from biodegradable polymers is affected by the pH of the polymer degradation environment.^{6,29,43–46} Polyanhydride degradation is known to be base-catalyzed.^{6,29} It is important to understand the release behavior of proteins from such polymers post injection, inside the host, because the intracellular pH tends to be more acidic (pH of 4.5–6) enabling the breakdown of phagocytosed particulates in endocytic compartments.⁴⁷ To this end, protein release kinetics were studied for five different chemistries of TRBSA-encapsulated CPH:SA nanospheres with the fluorescence-based protein detection assay in release media with three different buffered pH values: 7.3 (neutral), 6.0 (mildly acidic), and 4.3 (intracellular pH).⁴⁷ Because of the small sample size (~1 mg nanospheres) and the buffering capabilities of the release medium, it is highly unlikely that the polymer degradation will alter the pH of the release buffer. The high throughput technique allowed for concurrent, rapid protein quantification of a two-dimensional combinatorial library varying in nanosphere chemistry and pH of the release medium. The results are consistent with the base-catalyzed degradation mechanism of polyanhydrides, clearly demonstrating a reduced protein release as the pH of the release medium was lowered, as indicated in Figures 4–6. The pH level appears to more strongly affect the less hydrophobic (i.e., SA-rich) nanosphere chemistries, which is likely due to their more rapid degradation over the period of study. It is hypothesized that this effect would be evident for the more hydrophobic (CPH-rich) nanosphere chemistries if the study were carried out for a more extended period allowing for complete degradation of the polymer. While pH is not the only *in vivo*

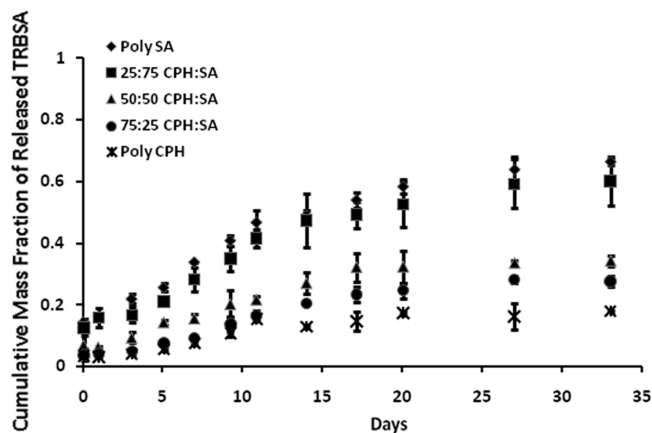


Figure 5. Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 6.0 as detected by the high throughput fluorescence assay. Error bars represent standard error and $n = 2$.

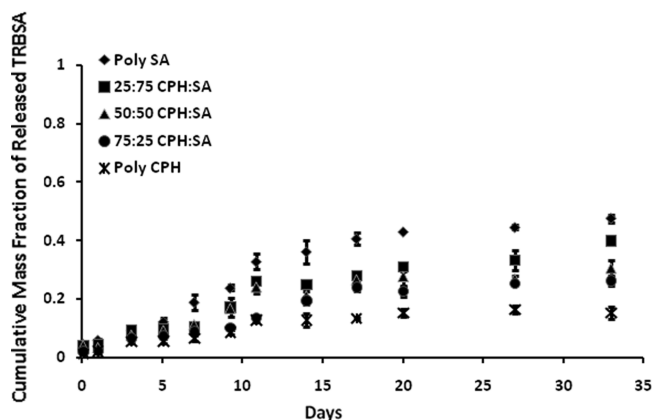


Figure 6. Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA nanospheres for one month at pH 4.3 as detected by the high throughput fluorescence assay. Error bars represent standard error and $n = 2$.

parameter that controls polymer degradation, this high throughput approach is amenable to simulate and study numerous other intracellular or extracellular phenomena (e.g., enzymatic degradation, serum protein adsorption).

Combinatorial Protein Release from Polyanhydride Films. The high throughput fluorescence method was also used to study the release of TRBSA from five linearly varying CPH:SA copolymer films in replicates of four, which was performed in parallel with the aforementioned nanosphere release. These studies were carried out for two reasons. First, there is interest in discerning the effect of device geometry (films vs nanospheres) on protein release kinetics. As mentioned before,^{3,42,48,49} polyanhydride devices of various geometries have been tested for in vivo applications, and the high throughput method provides a rapid way to simultaneously study the effects of polymer chemistry and device geometry. Second, it is important to demonstrate that the fluorescence technique is amenable to various device geometries. As expected, the films displayed a polymer chemistry-dependent protein release profile with the most hydrophobic (i.e., CPH-rich) chemistries releasing the protein the slowest (Figure 7). These results were in agreement with release profiles obtained with the micro-BCA assay (data not shown). The overall release of protein from the films

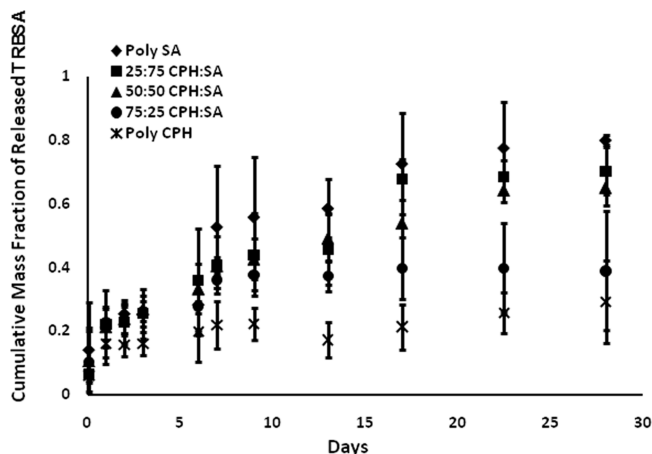


Figure 7. Cumulative mass fraction of TRBSA released from combinatorially fabricated CPH:SA films for 1 month at pH 7.3 as detected by the high throughput fluorescence assay. Error bars represent standard deviation and $n = 4$.

was lower and resulted in a smaller initial protein burst when compared with the nanospheres, which is likely due to the reduced polymer surface area exposed to the release buffer. The variances observed with the TRBSA release profiles from the films were higher than those with the TRBSA release profiles from the nanospheres. This observation can be attributed to the tendency of films, in some cases, to delaminate and break off into non-uniform pieces, thus exposing different amounts of surface area. As stated before, this technique is amenable to investigate drug/protein release from alternate geometries such as three-dimensional scaffolds used for tissue engineering^{50–52} or core-shell particles used for multidrug therapies.^{53,54}

Conclusions

The development of a highly sensitive fluorescent technique for the simultaneous detection of protein release from biodegradable polyanhydride nanospheres and films has enabled rapid evaluation of the effects of device geometry, polymer chemistry, and pH of release medium on the protein release kinetics. The film geometry, CPH-rich chemistries, and acidic release conditions all demonstrated the ability to significantly decrease protein release over their respective counterparts (nanosphere geometry, SA-rich chemistries, and neutral release conditions). Both the nanosphere and film systems released TRBSA protein in a sustained and polymer chemistry-dependent manner while the nanospheres also demonstrated a pH-dependent protein release. The high throughput fluorescence-based technique is amenable to other polymer and protein systems as well as alternate release environments which will allow for rational and rapid design of delivery devices. Finally, these findings contribute to the large body of evidence supporting the use of polyanhydrides as highly tunable biomaterials for the controlled delivery of drugs and proteins.

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